CHLOROFORM MODE OF ACTION ANALYSIS

October 6, 1999

This analysis follows the framework for mode of action analysis reviewed by the Science Advisory Board in January, 1999 and July, 1999 USEPA drafts of the revised *Guidelines for Carcinogen Risk Assessment.* The analysis is provided to assist in the Science Advisory Board October, 1999 consideration of the mode of action of chloroform.

Assessments of chloroform health risks referenced in support of this analysis are--

HRA (1998a): USEPA. Risk Assessment/Characterization of the Drinking Water Disinfection Byproduct Chloroform, TERA, November 4, 1998.

HRA (1998b): USEPA Health Risks to Fetuses, Infants and Children (Final Stage 1 D/DBP Rule). October 29, 1998.

ILSI (1997): International Life Sciences Institute (ILSI) An Evaluation of EPA's Proposed Guidelines for Carcinogen Risk Assessment Using Chloroform and Dichloroacetate as Case Studies: Report of an Expert Panel, November 1997.

1. Summary Description of Postulated Mode of Action

Studies in humans are inconclusive concerning whether chloroform is carcinogenic. Chloroform does result in an increased incidence of kidney tumors in male rats and, an increased incidence of liver tumors in male and female mice. Tumors are produced only at dose levels that result in cytotoxicity. These induced tumor responses are postulated to be secondary to **sustained** cytotoxicity and regenerative hyperplasia. Chloroform's carcinogenic effects in rodent liver and kidney are attributed to oxidative metabolism-mediated cytotoxicity in the target organs. Although chloroform undergoes both oxidative and reductive cytochrome P450-mediated metabolism, it is the oxidative (CYP2E1) metabolic pathway that predominates at low chloroform exposures. This oxidative pathway, produces highly tissue-reactive metabolites (in particular phosgene) that lead to tissue injury and cell death. It is likely that the electrophilic metabolite

phosgene causes cellular toxicity by reaction with tissue proteins and cellular macromolecules such as phospholipids, glutathione, free cysteine, histidine, methionine, and tyrosine. The liver and kidney tumors induced by chloroform depend on persistent cytotoxic and persistent regenerative cell proliferation responses. The persistent cell proliferation presumably would lead to higher probabilities of spontaneous cell mutation and subsequent cancer. The weight of the evidence indicates that a mutagenic mode of action via DNA reactivity is not a component of the chloroform carcinogenic process.

2. "Identification of key events"

There are essentially three key steps in the sequence of events that lead to chloroform induced tumorigenesis in the liver and kidneys of rodents. The first step is oxidative metabolism of chloroform in the target organs, kidney and liver. Numerous binding and metabolism studies (as described in ILSI, 1997 and HRA, 1998a) including a transgenic mouse model (Constan, 1999) provide support that chloroform is metabolized solely by the oxidative cytochrome P450 (CYP2E1) pathway at all doses studied. It is this pathway that would predominate at low exposures, and is rate-limiting to chloroform's carcinogenic potential. If it occurs, the reductive metabolism of which can lead to free radicals and tissue damage, is minor under normal physiological conditions. The next key step is the resultant cell death and cytotoxicity (histological evaluation) caused by the oxidative metabolites (with phosgene as the significant toxic intermediate). Regenerative cell proliferation follows the heptatoxicity and renal toxicity as measured by labeling index in mouse kidney and liver and rat kidney from chloroform-treated animals.

3. "Strength, consistency, specificity of association":

The association of cytotoxicity and tumor response is consistent among studies performed with chloroform. Only doses that cause toxicity and regenerative proliferation in the target sites--liver and kidney--produced tumors. Chloroform-induced tumors are consistently preceded by cytotoxicity and cell proliferation. In fact, one only finds evidence of persistent toxicity and regenerative proliferation in the rodent species, sex, and target organ where tumors arise, namely

liver and kidney. There is no evidence of chloroform-associated persistent toxicity and regenerative proliferation in other organs where tumors do not develop (Larson *et al*., 1996; Templin *et al*., 1996). This further reinforces the critical role that persistent toxicity and regenerative proliferation play in chloroform-induced liver and kidney neoplasia.

There are consistent observations of toxicity and tumor responses in target organs in which chloroform is most actively metabolized. Strong proof linking metabolism via CYP2E1 to toxicity and cell proliferation in the liver and kidney of mice is provided by experiments in an Sv/129 CYP2E1 null, wild type Sv/129, and $B6C3F_1$ mice (Constan, et al., 1997). In the wild type of each strain, exposure to 90 ppm chloroform for 4 consecutive days resulted in severe lesions and cell proliferation. With the same exposure, neither the cytotoxicity nor cell proliferation occurred in the CYP2E1 null mouse or in the wild type of either strains treated with the P450 inhibitor, ABT.

While there is no single, long-term bioassay for which all key events have been studied at the same time, the database provides numerous bioassays that show that cytotoxicity is critical to carcinogenicity and without it, there is no carcinogenicity. For example, in the $B6C3F_1$ mouse, corn oil gavage (bolus dosing) results in sustained cytolethality and a cell-proliferative response under the conditions that cause cancer. Similar daily doses administered in drinking water (lower continuous dose rate, but some average daily dose) did not induce cytolethality, cell proliferation, or cancer.

In the Osborne-Mendel and F-344 rat, single corn oil gavage doses of chloroform (0,10, 34, 90, 180, 477 mg/kg) produced cell proliferation in kidneys of both strains. In the Osborne-Mendel rat, this was a dose-dependent response at 10 mg/kg and above. In the F-344 rat, the increase was present only at 90 mg/kg and above. This is consistent with the observation that the Osborne-Mendel rat shows a cancer response under these conditions in long-term assays while the F344 rat does not, indicating a difference in strain sensitivity but the same mode of action.

A 2-year study was conducted in BDF_1 mice at 5, 30, or 90 ppm for 6 h/day, 5 days/week. Because these doses were nephrotoxic and lethal to male mice, the mice were conditioned by stepping up the dose gradually. (The mechanism of this adaptive response is not known.) Male mice showed kidney tumors at the top two doses; females showed no significant tumor response. In a 13 week study replicating the dose regimen of the 2-year study, observations were made of

cytotoxicity and labeling index. No increases were seen for either endpoint in females. Increases with both end points were seen in males at the top two doses as in the 2-year study. The effects were absent at 5 ppm--the dose at which there was also no significant tumor response in the 2-year study.

4. "Dose-response relationship":

Chloroform induced liver tumors in mice are only seen after bolus, corn oil dosing. Mouse liver tumors are not found following administration by other routes (drinking water and inhalation). Rat liver tumors are not induced by chloroform following either drinking water or corn oil gavage administration. Kidney tumors are found in mice exposed to chloroform via inhalation or in toothpaste preparations, and in rats when exposed via drinking water or corn oil gavage. Kidney and liver tumors develop only at doses that cause persistent cytotoxicity and regenerative proliferation, regardless of route of exposure or dosing regime. The overall dose-response for the cytotoxicity and cell proliferation responses is nonlinear. All key events and tumor effects depend on the dose-rate as shown by the difference in oil gavage versus drinking water administration. HRA 1998a, p 58, ILSI 1997, p. C46-52.

5. "Temporal relationship":

Cytotoxicity and regenerative proliferation are directly associated with carcinogenic doses. In short-term and long-term studies in mice and rats, cytotoxicity and cell proliferation are observed under conditions that result in kidney and liver tumor effects in long-term studies. For example, a re-evaluation of serial sacrifice data from the chloroform 2-year drinking water bioassay in Osborne-Mendel rats revealed a clear linkage between toxicity in the renal tubules and tumor development and showed that renal toxicity preceded tumor development (Hard and Wolf, 1999).

6. "Biological plausibility and coherence":

The theory that sustained cell proliferation to replace cells killed by toxicity, viral or other insult such as physical abrasion of tissues can be a significant risk factor for cancer is plausible and generally accepted (Correa, 1996). It is logical to deduce that sustained cytotoxicity and

regenerative cell proliferation may result in a greater likelihood of spontaneous mutations being perpetuated with the possibility of more or more of these resulting in uncontrolled growth. It may also be that continuous stimulus of proliferation by growth factors involved in inflammatory responses increases the probability that damaged cells may slip through cell cycle check points carrying DNA alterations that would otherwise be repaired. Current views of cancer processes support both of the above possibilities. There are no data on chloroform that allow the events that occur during cell proliferation to be directly observed. A high proliferation rate alone is not assumed to cause cancer; tissues with naturally high rates of turnover do not necessarily have high rates of cancer and tissue toxicity in animal studies does not invariably lead to cancer. Nevertheless, regenerative proliferation associated with persistent cytotoxicity appears to be a risk factor of consequence.

7. "Other modes of action":

Chloroform treatment-related tumorigenicity in liver and kidney of rodents is inconsistent among strains and inconsistent between rates of administration. Moreover, there are differences in response between species and sexes. Kidney tumor responses appear to vary with route of exposure, administration vehicle, and strain of rat, and liver responses are limited to the mouse, also varying by route, vehicle, and strain. This is not a typical pattern of response seen with mutagenic carcinogens.

The question whether chloroform or a metabolite is mutagenic has been tested extensively across different phylogenetic orders (i.e., bacterial, eukaryotic, and mammalian systems). Predominately negative results are reported in all test systems with no pattern of mutagenicity seen in any one system considered to be a competent predictor. Positive results appear sporadically in the data base, but are outnumbered by negative results in other tests in the same system. ILSI, 1997(p. C.29) considered results from 40 tests by the quantitative weight of evidence method for heterogeneous genetic toxicology databases (International Commission for Protection against Environmental Mutagens and Carcinogens-ICEMC (Lohman *et al*, 1992). This method scores relative DNA reactivity with a maximum positive score being +100, and maximum negative of -100. The maximum positive score obtained among 100 chemical databases has been +49.7

(triazaquone) and the maximum negative has been -27.7. The score for chloroform was -14.3.

Testing of chloroform in the p53 heterozygous knockout mouse shows no tumor effect (Gollapudi *et al.*, 1999). Heterozygous p53 males were dosed up to 140 mg/kg and females up to 240 mg/kg via corn oil gavage for 13 weeks. This model is known to respond most effectively to mutagenic carcinogens.

Products of oxidative and reductive metabolism of chloroform are highly reactive. Such species are unstable and will likely react with cytoplasmic macromolecules before reaching nuclear DNA. Such reactive species, e.g., phosgene. have not been evaluated separately for genetic toxicity, and because of reactivity, would not be amenable to study and would not likely be able to transport the cellular site of production to the nucleus. The many genotoxicity assay conducted on chloroform should have provided more evidence of any significant mutagenicity potential.

Comparative examination of both oxidative and reductive metabolism for structural analogues and chloroform has revealed that carbon tetrachloride, which is largely metabolized to a free radical via the reductive pathway, results in cell toxicity, not mutagenicity. Moreover, chloroform and carbon tetrachloride show very different patterns of liver toxicity (i.e., carbon tetrachloride's toxicity is more consistent with free radical production and chloroform's is not). For methylene chloride, glutathione conjugation results in mutagenic metabolites. When rat glutathione transferase gene copies are introduced into *Salmonella*, bromodichloromethane produces mutagenic metabolites; the fact that chloroform in this system did so only marginally and only at high toxic doses (Pegram *et al.*, 1997), provides support for a conclusion that the reductive pathway does not contribute to chloroform's toxicity and carcinogenicity.

In initiation-promotion studies, chloroform at the highest test dose of the drinking water bioassay, does not promote development of hepatic lesions in rats or two strains of mice, nor does it initiate or act as a co-carcinogen. Administered in oil, chloroform was a promoter in the rat liver in initiation-promotion protocols. These results are more consistent with the postulated mode of action than with any mutagenic potential.

8. "Conclusion":

The weight of the evidence provides support for the postulated mode of action for liver and

kidney tumors in rodents. Chloroform-induced tumors are only produced at dose levels that result in cytotoxicity and regenerative cell proliferation. A wide range of evidence across different species, sexes, routes of exposure clearly implicates oxidative CYP2E1 metabolism leading to persistent cytotoxicity, and regenerative cell proliferation as events which precede and are associated with the ultimate tumor result. Other modes of action have been well studied and are not supported by the evidence. The dose-response relationship for chloroform tumorigenesis by this mode of action will be nonlinear as it is dependent on biochemical and histopathological events that are nonlinear. The dose-response assessment would ideally be based on use of phosgene dosimetry because it marks the rate-limiting event of oxidative metabolism. Since the toxicokinetic modeling to support this phosgene approach is not currently available, the dose-response assessment should be based on the tumor precursor event of cytotoxicity to project a level of exposure that will be protective against the key event of cytotoxicity.

9. "Human relevance, including subpopulations" :

The cytochrome P450 oxidative metabolism which leads to oxidative damage and ensuing cell growth, involving basic tissue responses to cellular toxicity and death, are common to humans and rodents. No data exist on humans that would indicate lack of relevance of the rodent mode of action.

Data on human cancer (Ries et al., 1999) indicate that males and females are similar in types and incidence of hepatic cancer. In persons born in the US, hepatocelluar carcinoma is an uncommon tumor, being about 1.3% of incident cancers annually in the US. Alcohol consumption and viral infection are the two risk factors most often cited. Kidney cancer which is twice as common in men as women occurs at about 2% of incident cancers annually. Renal adenocarcinoma is the tumor type with few exceptions. The risk factors cited have been smoking, radiation, obesity, and pharmaceuticals; kidney cancer is not generally considered to be associated with occupational exposures.

US incidence of kidney and liver cancer in children

Wilms tumor accounts for 95% of the renal cancers in children and those younger than 20.

Wilms tumor is due to a germ cell mutation and is not related to adult onset of kidney cancer. Liver cancer is rare in children; 100-150 children younger than 20 are diagnosed with liver cancer yearly--about 1% of childhood cancers. Hepatoblastoma, a congenital cancer, is the more frequent liver neoplasm in infancy to 4 years, and thus is not related to adult onset of liver cancer. Less frequent is hepatocarcinoma which increases in proportion with age and is the prevalent adult tumor type. Recent studies have suggested an association of hepatoblastoma with prematurity and its treatment. For both liver and kidney cancer, there is limited or inconsistent evidence of association with specific parental occupational exposure to metals and organic chemicals and with maternal medication or other exposures. The incidence data do not suggest greater prevalence of cancer at either site in children as compared with adults, but the histology in adults is more likely to be carcinoma which is less associated with congenital anomalies or genetic conditions.

Liver toxicity in younger versus older rodents

In a 2-generation study , CD-1 (ICR)BR mice were exposed to chloroform *in utero*, during lactation, and then by gavage as young mice through "young" adulthood. The only liver effect observed was mild to moderate liver histopathology (degeneration of centrilobular hepatocytes, accompanied by occasional single cell necrosis) in females at 41 mg/kg-day, the only dose at which systemic effects were evaluated. Thus, the only dose tested in this study, 41 mg/kg-day, was a LOAEL for liver histopathology. (HRA 1998a, p. 18, HRA, 1998b). No effects of chloroform on reproductive function were identified (NTP, 1988). Oral developmental toxicity studies have found decreased fetal weight (Thompson *et al*., 1974) and inhalation developmental studies have found an increased incidence of delayed ossification in Wistar rats (Baeder and Hofmann, 1991), but these effects occurred at doses above those causing hepatotoxicity.

Metabolism of chloroform in fetuses, infants and children compared to adults (HRA 1998a, p. 20)-- *implications for quantitative dose-response relationship.*

Metabolism of chloroform is essential to its toxicity (HRA, p.22). Moreover, metabolism by cytochrome P450 CYP2E1 is required for toxicity to both liver and kidney of B6C3F1 and Sv/129 male mice (Constan *et al*., 1997). Because of the role of CYP2E1 in chloroform's mode of carcinogenic action, it is important to evaluate CYP2E1 activity in tissues of the young compared to adults to determine whether the young might respond at a lower dose than adults.

The status of CYP2E1 in fetuses remains unclear, with conflicting studies. Most of the existing studies indicate that this enzyme is expressed in human adults but not in human fetuses, even when measured using sensitive assays (reviewed in Hakkola *et al*., 1998). In these studies, levels of both CYP2E1 protein and of the associated enzyme activity were undetectable before birth, but rose rapidly shortly after birth, due to stabilization of the CYP2E1 protein. However, at least three studies indicate CYP2E1 is expressed in fetal liver or cephalic tissue (Boutelet-Bochan *et al*., 1997; Carpenter *et al*., 1996; Vieira *et al*., 1996). Boutelet-Bochan *et al*. (1997) detected low levels of CYP2E1 mRNA transcription in human fetal brains (gestation days 52-117, or 7-17 weeks), and levels tended to increase with gestational age. However, transcription was detected only using a very sensitive assay (reverse transcriptase-polymerase chain reaction - RT-PCR) or the moderately sensitive RNase protection assay. Transcription in fetal liver was much lower, and was detectable in only two of six samples. Also using the RNase moderately sensitive technique, Carpenter *et al*. (1996) found transcription of CYP2E1 mRNA in the liver of human fetuses at 19-24 weeks gestation, but not at 10 weeks gestation. Fetal liver microsomes could metabolize the CYP2E1 substrate ethanol, but at a rate only 12-27% of adult liver microsomes. At least most of the observed activity was specific to CYP2E1, since it was inhibited by an anti-CYP2E1 antibody. Like adult hepatocytes, fetal hepatocytes exposed to ethanol had induced levels of CYP2E1. Vieira *et al*. (1996) found that CYP2E1 protein could not be detected immunochemically in fetal human liver, and there was only minimal evidence of CYP2E1 mRNA or CYP2E1 activity in fetal liver microsomes. (The difference in assay results may be due to differences in sensitivity, or to cross-reaction of CYP1A1 activity.) The authors found, however, that CYP2E1 protein levels rise rapidly in the first few hours after birth, with a slow increase in protein levels and in CYP2E1 RNA

levels during childhood.

Thus, the overall human data show that if CYP2E1 activity exists in human fetuses, levels are much lower than those in adults. Regardless of fetal CYP2E1 expression, the enzyme is rapidly induced upon birth. For this reason, children would be expected to be capable of chloroform metabolism, although the amount of CYP2E1 may be less than that present in the adult. Overall, the data on CYP2E1 activity provide no evidence to suggest that children are more susceptible than adults.

The animal studies of developmental CYP2E1 regulation provide uniform evidence of the rapid induction of this gene soon after birth (Song *et al*., 1986; Umeno *et al*., 1988; Schenkman *et al*., 1989; Ueno and Gonzalez, 1990). The idea that the enzyme activity peaks before weaning with a gradual decrease to adult levels suggested by some scientists, however, has not been consistently reported in the three studies which compared expression over this period of time.

For example, Schenkman *et al*. (1989) indicate that CYP2E1 protein is present in low levels in neonates, rises to a peak level at age 2 weeks, and subsequently decreases to adult levels by puberty. Analysis of protein levels quantified from western blots showed a maximum at 2 weeks with decreasing levels at 4 and 12 weeks. The protein level at 12 weeks was approximately 50% of the level at 2 weeks. The authors did not provide a statistical analysis of this result, but it appears from the error bars that the 2-week and 12-week levels (but not 4 week levels) were significantly different.

Song *et al*. (1986) conducted a similar analysis and reported a rapid transcriptional induction of CYP2E1 within 1 week following birth which remained elevated throughout 12 weeks. The authors did not quantify the western blots, but visual inspection indicates a small decline in protein levels by 12 weeks. However, in this same study, enzyme activity gradually increased over time, reaching a maximum at adulthood.

Ueno and Gonzalez (1990) showed that extracts from 3 day old and 12 week old rat liver, but not fetal or newborn rat liver, were able to generate significant CYP2E1 transcription in vitro. The ability of the extract to drive transcription of CYP2E1 was slightly greater at 12 weeks.

If the two-fold increase in CYP2E1 induction in animals were verified, its importance in terms of chloroform toxicity would depend on the dose. Under low dose conditions (for example,

much lower than the Km) it is possible that an increase in the level of enzyme would not have any effect on active metabolite formation since the amount of chloroform, and not CYP2E1, would control the rate of the enzyme activity. On the other hand, under saturating doses of chloroform, all the available enzyme would be active, thus a two-fold increase in CYP2E1 could result in greater activation of the compound. Additional analysis of the expected dose relative to the levels of enzyme could help elucidate the potential for these differing scenarios to occur.

Taken together, these animal studies do not provide conclusive evidence of an early period of increased enzymatic activity in young animals when compared with adults. While the animal data remain unclear regarding the potential for a period of increased CYP2E1 activity above that in the adult, for humans, a gradual increase of CYP2E1 activity throughout childhood with a maximum level at adulthood, as described by Hakkola *et al*. (1998), appears to be the most likely situation.

Conclusion

The evidence supports similarity of potential response in children and adults. The basic biology of toxicity caused by cell damage due to oxidative damage is expected to be the same. There is nothing about the incidence and etiology of liver and kidney cancer in children to indicate that they would be inherently more sensitive to this mode of action. Most importantly in this case, children appear to be no different quantitatively in ability to carry out the oxidative metabolism step for the induction of toxicity and cancer and may, as fetuses, be less susceptible.

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